Hepatitis B, Aflatoxin B_1, and *p53* Codon 249 Mutation in Hepatocellular Carcinomas from Guangxi, People's Republic of China, and a Meta-analysis of Existing Studies[1]

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Abstract

The incidence of hepatocellular carcinomas (HCC) varies widely worldwide, with some of the highest incidence rates found in China. Chronic infection with the hepatitis B virus (HBV) and exposure to aflatoxins in foodstuffs are the main risk factors. A G to T transversion at codon 249 of the *p53* gene (249^{ser}) is commonly found in HCCs from patients in regions with dietary aflatoxin exposure. Because HBV infection is often endemic in high aflatoxin exposure areas, it is still unclear whether HBV acts as a confounder or as a synergistic partner in the development of the 249^{ser} *p53* mutation. Our report has two aims. First, we contribute data on HCCs from southern Guangxi, a high aflatoxin exposure area. Using DNA sequencing, we found that 36% (18 of 50) of tumors had a 249^{ser} mutation. Also, 50% (30 of 60) were positive for p53 protein accumulation and 78% (28 of 36) were positive for HBV surface antigen, as detected by immunohistochemistry. Second, we present a meta-analysis, using our results along with those from 48 published studies, that examines the interrelationships among aflatoxin exposure, HBV infection, and *p53* mutations in HCCs. We used a method that takes into account both within-study and study-to-study variability and found that the mean proportion of HCCs with the 249^{ser} mutation was positively correlated with aflatoxin exposure ($P = 0.0001$). We found little evidence for an HBV-aflatoxin interaction modulating the presence of the *p53* 249^{ser} mutation or any type of *p53* mutation.

Introduction

The incidence of HCC[3] varies widely worldwide. Among males, the highest incidence rates are found in eastern Asia, particularly in China where HCC is the third most common cause of cancer death (1). Chronic infection with the HBV is the strongest risk factor for HCC worldwide (2–4). However, populations with similar prevalence of HBV infection have different incidence of HCC (4), suggesting the presence of other important risk factors. Aflatoxins, a group of mycotoxins produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus*, are established human hepatocarcinogens (5–9) and are well-known HCC risk factors when present in foodstuffs (3, 10).

The high-risk areas in eastern China include the Guangxi Autonomous Region. Fusui County in southern Guangxi has a standardized rate of primary liver cancer among men of 120/100,000 population/year, a rate 35 times higher than that in the United States (3). HCC accounts for 50% of all of the cancer deaths in men and 25% of all of the cancer deaths in women in this region (11). A cohort study done in this area found a positive linear relationship for AFB_1 levels in foodstuffs and mortality attributable to HCC (3). The levels of AFB_1 were estimated to be as high as 2575 ng/kg/day (3), in contrast to the estimated 3 ng/kg/day exposure in the United States (12). Infection with HBV was significantly associated with HCC mortality, with 91% of HCC deaths occurring in HBV-positive subjects (3).

The tumor suppressor gene *p53* is the most commonly mutated gene in human cancers (13). A G to T transversion at the third position of codon 249 of the *p53* gene (249^{ser}) is commonly found in HCC from patients in regions with dietary aflatoxin exposure (14–24). *In vitro* studies (25–27) have supported this finding, showing that AFB_1 can induce this mutation. The biological activity of the 249^{ser} mutant p53 protein remains undetermined. However, experiments (28) conducted with a murine p53 protein carrying the codon 249 homologous mutation (*p53*ser246) showed that the mutant protein could transform cells in culture and was defective in its transcription activation function. Studies done worldwide (reviewed in Refs. 29, 30) suggest that the frequency of the *p53* 249^{ser} mutation in HCC correlates with the exposure level of AFB_1 in the underlying population. However, relatively few studies have been

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; AFB_1, aflatoxin B_1; HBsAg, HBV surface antigen; CI, confidence interval.

conducted in high aflatoxin exposure areas, and many have been small.

Some epidemiological (10, 24, 31, 32) and animal (33, 34) studies have found evidence for an HBV-aflatoxin interaction in hepatocarcinogenesis (reviewed in Ref. 30). Several mechanisms have been proposed to explain this interaction. The increase in cellular proliferation induced by HBV (35) could increase the probability for clonal expansion of an existing aflatoxin induced-*p53* 249^{ser} mutation. An increase in levels of aflatoxin metabolism enzymes (*e.g.,* P450 enzymes) has been described for HBV transgenic mice and has been postulated as a mechanism for interaction (36). The HBVx protein, encoded by HBV, interferes with the nucleotide excision repair pathway (37), which cells use to repair aflatoxin-DNA adducts. Thus, the presence of the HBVx protein could increase the frequency of aflatoxin-induced mutations (37). Lastly, HBV infection could increase oxidative stress, which could lead to an increase in *p53* mutations (38).

Epidemiological studies of the interrelationship among aflatoxin exposure, HBV status, and the presence of the *p53* 249^{ser} mutation could help clarify whether HBV acts as a confounder or as a synergistic partner with aflatoxin. One study (24) in Taiwan found that, in the presence of aflatoxin, the *p53* 249^{ser} mutation was significantly more frequent among HBV-positive than -negative subjects. Other studies found similar but not significant trends (18, 32, 39) or no differences at all (19, 21, 23, 40–46). The main limitation in investigating possible HBV-aflatoxin interaction is that in high exposure areas HBV infection is generally endemic, and few HBV-negative individuals are available for study.

Our report has two aims. First, we contribute additional data on *p53* 249^{ser} mutation and HBV infection in HCC from southern Guangxi. This area has one of the highest aflatoxin exposure rates in the world and has provided valuable data establishing the role of aflatoxin and HBV in HCC (3, 11, 47), but tumors from this region have never been analyzed for 249^{ser} mutations. Second, we present a meta-analysis using our results along with those from 48 published studies to examine the interrelationships among aflatoxin exposure, HBV infection, and *p53* mutations in HCC, focusing on a possible HBV-aflatoxin interaction in producing 249^{ser} mutations.

Materials and Methods

HCCs from Southern Guangxi

Patients and Samples. We analyzed 64 paraffin-embedded tissue blocks from patients with liver resection for HCC from southern Guangxi, China. All of the patients were permanent residents of Nanning City and surrounding counties.

Detection of 249^{ser} Mutation. We used a laser capture microdissection instrument (Arcturus Engineering, Inc.) to remove non-neoplastic tissue from several consecutive 10–15-μm-thick sections of each HCC paraffin-embedded tumor block. We resuspended the microdissected samples in 50–200 μl of digestion buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20, and 0.04% proteinase K (Sigma Chemical Co., St. Louis, MO) and incubated them at 55°C overnight. After proteinase K digestion, samples were boiled for 10 min and centrifuged to pellet tissue debris. The supernatant containing DNA was aliquoted and stored at −20°C until use. Given the old age and long fixation time of the tumor blocks, we were unable to obtain high quality DNA from many samples, which prevented us from performing a reliable systematic analysis of the entire *p53* gene. We focused on the analysis of codon 249 by amplifying a region of exon 7 surrounding codon 249 using forward (5′-AACAGTTCCTGCATGGGCGG-3′) and reverse (5′-CCAGTGATGATGGTGAGG-3′) primers. PCR reactions were carried out in a PE 9700 (Perkin-Elmer, Foster City, CA) with an initial denaturation step of 2 min at 94°C followed by 40 cycles of 94°C for 30 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min. The 58-bp long PCR products were purified using exonuclease I and shrimp alkaline phosphatase (Amersham Life Sciences, Cleveland, OH). Direct sequencing was performed using the Amersham Thermosequenase Radiolabeled Terminator Kit (Amersham Life Sciences) with the same primers used for PCR amplification. Mutations were confirmed in both forward and reverse directions.

Immunohistochemistry Analysis. For p53 protein detection, we deparaffinized tumor sections and stained them using a Ventana NexEs automated immunostainer (Tucson, AZ) using a rabbit anti-p53 primary antibody (NCL-p53-CM1p; Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom). For detection of HBsAg, we deparaffinized tumor sections and quenched them with H_2O_2 for 15 min. We incubated slides first in 5% normal horse serum for 20 min at room temperature and then with a 1:100 dilution of mouse monoclonal primary antibody against HBsAg for 1 h at room temperature (Novocastra Laboratories). After rinsing, slides were incubated with a secondary antibody from the Vector Mouse Elite kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, rinsed again, and incubated with labeled antibodies following kit instructions. After a final rinsing, slides were stained with 3,3′-diaminobenzidine and counterstained with hematoxylin for detection. For both proteins, appropriate positive and negative controls were used. A pathologist, blinded to HBV or *p53* status, analyzed all of the immunostained tumors. Where possible, non-neoplastic areas surrounding the tumors were also analyzed. For p53 staining, tumors were classified as positive or negative for the presence of nuclear staining above background. For HBV analysis, tumors were scored positive or negative for the presence of cytoplasmic staining.

Statistical Analysis. To examine possible associations between pairs of dichotomous outcomes, we analyzed 2×2 tables using Fisher's exact test (48). Outcomes included the subject's sex as well as the presence or absence of 249^{ser} mutation, of p53 protein accumulation, and of detectable HBsAg. We compared mean age at cancer diagnosis among groups defined by combinations of outcome variables using ANOVA methods (48).

Meta-Analysis. We restricted our review to studies published in peer-reviewed journals before March 2000, plus the study presented here. To find studies, we searched Medline using search strings that used combinations of the words: *p53* mutation, HCC, liver cancer, aflatoxin, HBV, or codon 249. We also looked at articles cited in those found via Medline.

Our goal was to analyze the proportion of tumors carrying *p53* mutations and to see how those proportions changed with the aflatoxin exposure level in the study area and with HBV infection in the patient. To be included in our analysis, a study had to present the total number of tumors analyzed and individual information on *p53* mutations obtained by direct DNA analysis. A number of studies, like our own, focused on 249^{ser} mutations, either by RFLP or by direct sequencing, and did not screen for mutations in the rest of the gene. These studies are used in some analyses. To include a study in our analysis of all of the *p53* mutations, we required it to have sequenced at least exons 4 through 8. HBV infection status was assessed in different studies using serum samples, tumors, or both by a variety of methods, including immunostaining, PCR to detect inte-

grated viral genome, ELISA, RIA, or Southern blots. In those studies where several techniques were used, we considered a subject positive if at least one technique showed positive results. To include a study in our analysis of whether HBV infection modified the effect of aflatoxin exposure on mutation frequency, we required cross-classified information on HBV infection and *p53* mutation status. Finally, we required each study to provide sufficient information on the geographic origin of the tumor samples so that we could assess aflatoxin exposure. We classified study locations as having high, moderate, or low aflatoxin exposure based on work by Shen and Ong (29). They reviewed various studies and classified each according to either the quantity of aflatoxin in consumed food and/or determination of biomarkers (AFB_1-DNA or protein adducts) in human tissue samples from each region using published information. We performed similar assessments for study locations that they did not classify.

We calculated several response variables for each study. These included the proportion of tumors that had any mutation in p53, the proportion of tumors that had a 249^{ser} mutation, and the proportion of tumors with a 249^{ser} mutation among those that had any p53 mutation. We also calculated the proportion of patients that had HBV infections among all of the patients. To examine possible HBV-aflatoxin interactions, we focused on the difference in the proportion of tumors with a given mutation between patients with HBV infection and those without it and looked at whether that difference might change with aflatoxin level. We also looked for interaction on the log odds ratio scale by applying a modified empirical logistic transformation (49) to the proportions and analyzing whether differences in the transformed proportions (*i.e.,* empirical log odds ratios) changed with aflatoxin level. As a measure of interaction for this analysis, we preferred the difference in proportions to the empirical log odds ratios, primarily because with the latter measure observed proportions of zero must be excluded from the analysis, eliminating studies; *e.g.,* if the observed proportions of 249^{ser} mutations were zero in both HBV-positive and HBV-negative patients in a given study, we included that study in the analysis of differences in proportions (zero minus zero is zero) but not in the analysis on the log odds ratio scale (because zero divided by zero is indeterminate).

Each response variable was analyzed with a random effects model (50) incorporating study as a random effect and using aflatoxin exposure as a categorical covariate. We fitted these models using the MIXED procedure of Statistical Analysis System statistical software (SAS Institute, Inc., Cary, NC) following an approach outlined by Normand (51). This approach takes account of study-to-study heterogeneity in the underlying mean response that might arise, for example, from different populations under study or from different laboratory methods. The resulting estimates of mean response at each exposure level are weighted to take account of both within-study and study-to-study sources of variation and to accommodate the differing sample sizes in each study. When a proportion of zero or one is observed, the usual within-study estimate of the variance for such an observation is zero. Consequently, we examined the sensitivity of our conclusions to minor perturbations in within-study estimated variance. We also checked whether individual studies exerted undue influence on our conclusions by dropping one study at a time and reanalyzing the remainder.

Results

HCCs from Southern Guangxi. Patients ranged in age from 20 to 72 years. Most were men (77%). In 14 (22%) of the 64 samples, we were not able to obtain PCR products because of poor quality DNA. Of the remaining samples, we found that 18 (36%) had a G to T transversion at the third base of codon 249 of the *p53* gene (249^{ser}), whereas 32 (64%) were wild type. We observed no mutations in other codons of this small region of exon 7. For 22 (44%) of these samples, we performed independent DNA extractions and duplicate analysis to confirm the results and observed 100% concordance between these independent determinations. We found no differences in the frequency of 249^{ser} mutation between females (33%) and males (37%; $P = 1.00$). Patients with 249^{ser} mutation showed no statistically significant difference in mean age from those without the mutation (39 ± 8.3 *versus* 44 ± 13; $P = 0.22$).

Thirty samples (50%) showed accumulation of p53 protein in tumor cells. Four samples could not be analyzed. For the 48 samples with both DNA mutation and immunohistochemistry results, we saw concordance between protein accumulation and mutation in 30 samples, whereas 18 were nonconcordant. Among nonconcordant samples, three had 249^{ser} mutation without p53 protein accumulation, and 15 had wild-type codon 249 but positive immunohistochemistry. We found no statistically significant difference in the proportion of tumors with positive p53 staining between females (43%) and males (52%; $P = 0.76$). The mean age did not differ between patients positive (42 ± 13) and negative (42 ± 11) for p53 immunohistochemistry ($P = 0.99$).

For the analysis of HBV infection, we only included the 36 tumors that had non-neoplastic tissue available for staining because previous reports (52, 53) indicated that the majority of liver tumors tend to lose the HBV surface antigen, giving false-negative results. We excluded seven samples because poor tissue quality precluded their analysis. Twenty-eight tumors (78%) were positive for HBsAg, whereas eight (22%) were negative. Among the 28 positive tumors, 23 (82%) expressed the HBV antigen only in the non-neoplastic liver tissue, and 5 (18%) expressed it in both the neoplastic and non-neoplastic tissue. HBsAg status did not differ by gender ($P = 1.00$). Mean age at cancer diagnosis did not differ by HBsAg status (42 ± 11 *versus* 48 ± 15; $P = 0.20$).

Accumulation of p53 protein was detected in 17 (61%) of the 28 HBsAg-positive patients, compared with 2 (22%) of the 9 HBsAg-negative patients ($P = 0.06$). The 249^{ser} mutation was found in 9 (36%) of the 25 HBsAg-positive patients, compared with 2 (40%) of the 5 HBsAg-negative patients ($P = 1.00$). Mean age at diagnosis did not differ among four groups of patients defined by cross-classifying HBsAg status with presence or absence of p53 accumulation in their tumors. Mean age at diagnosis did appear to differ, however, among four groups of patients defined by cross-classifying HBsAg status with codon 249 mutation status of their tumors ($P = 0.07$). In particular, HBsAg-positive patients whose tumors had the 249^{ser} mutation had lower mean age at diagnosis than HBsAg-negative patients whose tumors had wild-type *p53* (40 ± 6.8 *versus* 58 ± 1.5 years old, respectively).

Meta-Analysis. In all, 49 studies were available for meta-analysis (Table 1), 48 from the literature plus the current study, although not all of these studies contained data to address each question. We proceeded in two phases: we considered possible associations between *p53* mutations and aflatoxin level ignoring HBV infection and, after that, we examined the role of HBV infection.

Table 1 Studies used for meta-analysis[a]

Location (reference)	249^{ser}/total number of HCC	Any *p53* mutation/total No. of HCC	HBV+/total No. of HCC	249^{ser}+/HBV+	249^{ser}+/HBV−	Any *p53*+ /HBV+	Any *p53*+/ HBV−
High aflatoxin exposure regions							
Guangxi, China (53)	na[b]	na	27/30	na	na	na	na
Guangxi, China (current study)	18/50	na	28/38	8/24	2/5	na	na
Jiang-Su, China (41)	9/16	11/16	12/14	8/12	1/2	8/12	2/2
Mozambique (22)	8/15	na	15/15	8/15	0/0	na	na
Mozambique (42)	8/16	9/13	14/16	7/14	1/1	8/12	1/1
Mozambique (60)	5/6	na	7/7	5/6	0/0	na	na
Qidong, China (16)	8/16	8/16	na	na	na	na	na
Qidong, China (18)	21/35	na	34/36	21/33	0/2	na	na
Qidong, China (19)	9/20	9/20	16/20	8/16	1/4	8/16	1/4
Qidong, China (20)	13/25	15/25	25/25	13/25	0/0	15/25	0/0
Qidong, China (61)	7/14	7/14	14/14	7/14	0/0	7/14	0/0
Senegal (23)	10/15	na	13/15	8/13	2/2	na	na
Southern Africa (21)	3/10	5/10	8/9	3/8	0/1	5/8	0/1
Tongan, China (40)	7/21	na	13/16	6/13	1/3	na	na
Moderate aflatoxin exposure regions							
Durban, South Africa (62)	2/24	na	na	na	na	na	na
Hong Kong, China (43)	2/26	6/26	28/31	2/24	0/2	6/24	0/2
Mexico (46)	3/16	na	11/20	1/6	1/9	na	na
Shanghai, China (19)	1/18	3/18	15/18	1/15	0/3	3/15	0/3
Shanghai, China (32)	10/52	na	37/48	9/37	0/11	na	na
Shanghai, China (44)	1/12	2/12	7/12	0/7	1/5	2/7	1/5
Shanghai, China (61)	3/10	5/10	10/10	3/10	0/0	5/10	0/0
Taiwan (24)	12/102	27/102	78/105	12/75	0/25	22/75	4/25
Taiwan (39)	4/61	20/61	41/61	4/41	0/20	15/41	5/20
Taiwan (63)	3/38	16/38	na	na	na	na	na
Taiwan (64)	0/13	3/13	17/20	0/13	0/0	3/13	0/0
Taiwan (65)	3/15	na	na	na	na	na	na
Thailand (45)	1/15	2/15	7/13	1/7	0/6	2/7	0/6
Transkei, South Africa (22)	1/12	na	12/12	1/12	0/0	na	na
Xian, China (44)	1/45	2/11	24/39	0/24	1/15	1/3	1/7
Low aflatoxin exposure regions							
Alaska, United States (66)	0/13	0/13	12/14	0/12	0/1	0/12	0/1
Europe (67)	0/14	1/14	4/14	0/4	0/10	0/4	1/10
Europe (68)	1/19	6/19	7/22	1/7	0/12	2/7	4/12
Germany (69)	0/13	2/13	4/13	0/4	0/9	0/4	2/9
Germany (70)	0/20	2/20	4/20	0/4	0/16	1/4	1/16
Great Britain (71)	0/19	2/19	6/19	0/6	0/13	0/6	2/13
Italy (72)	0/20	6/20	12/20	0/12	0/8	5/12	1/8
Japan (73)	0/60	na	9/55	0/9	0/46	na	na
Japan (65)	0/6	na	na	na	na	na	na
Japan (74)	0/52	18/52	41/52	0/41	0/11	10/41	8/11
Japan (75)	0/43	7/43	na	na	na	na	na
Japan (76)	0/53	17/53	7/53	0/7	0/46	3/7	14/46
Japan (77)	0/20	3/20	8/20	0/8	0/12	1/8	2/12
Japan (78)	4/128	na	na	na	na	na	na
Japan (79)	3/34	10/34	na	na	na	na	na
Japan (80)	0/41	11/41	10/41	0/9	0/27	2/9	9/27
Northern China (41)	1/15	4/15	5/9	1/5	0/4	3/5	1/4
Singapore (81)	0/44	12/44	31/44	0/31	0/13	10/31	2/13
Singapore (52)	na	na	29/46	na	na	na	na
United States (82)	4/37	na	5/37	3/5	1/32	na	na

[a] For the analysis of *p53* 249^{ser} mutation or any *p53* mutation and HBV status, we could only used tumors that had data for these two factors. Therefore, the total number of mutations within these analyses may not be the same as the total number of mutations observed when ignoring HBV status.
[b] na, data not available.

First, we investigated whether aflatoxin exposure level in a study area was associated with the proportion of tumors carrying any mutation in *p53* (Table 2).

The proportion of tumors carrying any mutation in *p53* showed a broad range across studies within each aflatoxin level: 45–69%, 13–50%, and 0–35% for high, moderate, and low levels, respectively. Our analysis showed that the mean proportion of tumors with some *p53* mutation changed with aflatoxin ($P = 0.0001$), with the highest weighted average in high aflatoxin areas (57%; Table 2). We saw a significant difference in mean frequency of *p53* mutations between high and moderate areas and between high and low areas.

Next, we looked at whether the proportion of tumors with a G to T mutation at the third position of *p53* codon 249 (249^{ser}) varied with aflatoxin exposure (Table 2). We found that these proportions exhibited variations within each afla-

Table 2 Variables analyzed in meta-analysis

	Aflatoxin exposure	No. of studies	No. of tumors	Mean proportion of tumors percentage (95% CI)[a]
Presence of any *p53* mutation among all of the tumors[b]	Low	15	420	19 (14–25)
	Medium	10	306	26 (18–34)
	High	7	114	57 (46–68)
	Total	32	840	
Presence of *p53* 249^{ser} mutation among all of the tumors	Low	19	651	2 (0–6)
	Medium	15	459	9 (4–14)
	High	13	259	50 (42–58)
	Total	47	1369	
Presence of a *p53* 249^{ser} mutation among tumors with p53 mutations	Low	14	101	2 (0–10)
	Medium	10	86	27 (13–40)
	High	7	64	92 (80–100)
	Total	31	251	
Presence of non-249^{ser} *p53* mutations among all of the tumors without a mutation in codon 249	Low	15	415	19 (13–24)
	Medium	10	278	18 (11–25)
	High	7	57	5 (0–14)
	Total	32	750	
Presence of HBV infection among all of the tumors[c]	Low	16	479	41 (31–51)
	Medium	12	389	78 (67–89)
	High	13	255	91 (80–100)
	Total	41	1123	
Difference between the presence of any *p53* mutation among HBV+ and HBV− tumors	Low	13	332	−3 (−16–9)
	Medium	7	240	18 (1–35)
	High	4	56	−1 (−25–23)
	Total	24	628	
Difference between the presence of *p53* 249^{ser} mutation among HBV+ and HBV− tumors	Low	15	424	3 (−6–11)
	Medium	9	332	8 (−4–20)
	High	8	153	8 (−9–24)
	Total	32	909	

[a] Weighted to reflect both within-study and between-study variation as well as the sample size in each study.
[b] Mutations detected by sequencing exons 4–8 of *p53* gene.
[c] HBV status detected by a variety of techniques as described in "Materials and Methods."

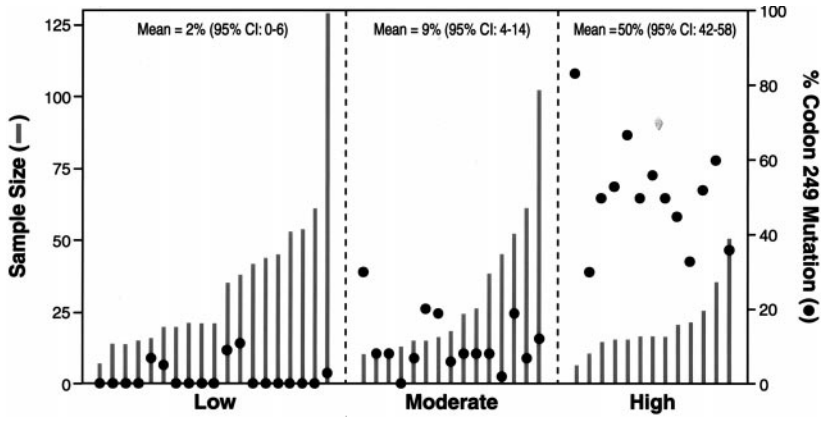

Fig. 1. Frequency of *p53* 249^{ser} mutation and sample size by aflatoxin level. *Bars*, sample size of each study referred to the *left* vertical axis. ●, observed prevalence of each mutation in each study referred to the *right* vertical axis. The horizontal axis orders each study contributing information on *p53* 249^{ser} mutations from smallest to largest in sample size within each aflatoxin level. The mean frequency (and CI) were derived from our meta-analysis.

toxin exposure level, ranging from 30–83%, 0–30%, and 0–11%, respectively (Fig. 1). The mean proportion of tumors having 249^{ser} mutations changed with aflatoxin exposure ($P = 0.0001$) and was significantly larger in high aflatoxin areas (50%). Mean proportions did not differ significantly between the moderate (9%) and low (2%) aflatoxin areas.

The observed decrease in the proportion of 249^{ser} mutations with decreasing aflatoxin exposure could reflect a general decrease in all of the *p53* mutations or a decrease in this specific mutation alone. A comparison of mean proportions suggested that tumors with a 249^{ser} mutation represented a majority of the tumors with *p53* mutations in high aflatoxin areas but a minority in moderate or low aflatoxin areas. We examined this issue directly by analyzing the fraction of all of the tumors carrying *p53* mutations that had a mutation in 249^{ser}. The mean proportion of tumors with a 249^{ser} mutation among tumors with any *p53* mutation was strongly associated with level of aflatoxin exposure ($P = 0.0001$). At the high exposure level, on average 92% of tumors with *p53* mutations were altered at codon 249 (Table 2). The corresponding mean proportions at the moderate and low exposure levels were 27% and 2%, respectively. All of

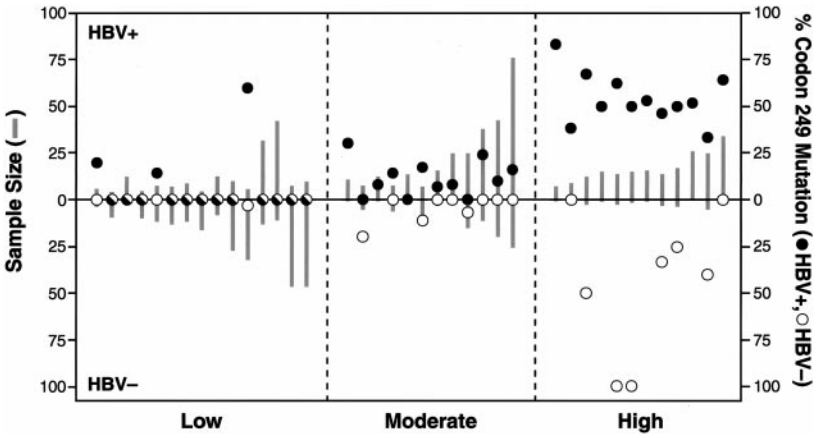

Fig. 2. Frequency of *p53* 249^{ser} mutation and sample size by aflatoxin level for HBV-positive and HBV-negative subjects separately. Vertical axes increase upward for HBV-positive and downward for HBV-negative subjects. *Bars,* sample size of each study (*left* vertical axis). ● for HBV positive and ○ for HBV negative represent the observed prevalence of the mutation in each study (*right* vertical axis). The horizontal axis orders each study contributing information on *p53* 249^{ser} mutations from smallest to largest in total sample size within each aflatoxin level. Note that three moderate aflatoxin studies and four high aflatoxin studies observed no HBV-negative subjects and could not be used in our analyses.

the pairwise comparisons between aflatoxin levels were statistically significant. Thus, at higher aflatoxin levels, tumors with 249^{ser} mutations represented a sharply increasing fraction of the tumors carrying *p53* mutations.

The previous analyses indicate that the increase in *p53* mutations with increasing aflatoxin exposure mainly reflects an increase in 249^{ser} mutations. However, they do not indicate whether the frequency of mutations at *p53* sites other than 249^{ser} also change with aflatoxin exposure. To address this issue, we eliminated all of the tumors with 249^{ser} mutations from the analysis (both numerator and denominator) and examined whether the proportion of *p53* mutations in the remaining tumors changed with aflatoxin level (Table 2). We found evidence that aflatoxin exposure was associated with the proportion of tumors with non-249^{ser} mutations ($P = 0.035$). Omission of any one of three studies from high exposure areas that found only 249^{ser} mutations (*i.e.,* estimated proportion of zero) rendered the association nonsignificant ($P > 0.15$), whereas omission of a single low exposure study that reported no non-249^{ser} *p53* mutations enhanced statistical significance. Consequently, in our view, how aflatoxin exposure may relate to the frequency of *p53* mutations at sites other than codon 249 remains an open question.

The mean proportion of HBV-positive cases among all of the cases was associated with aflatoxin exposure level ($P = 0.0001$; Table 2). That proportion decreased from 91% in high exposure regions to 76% and 41% in moderate and low exposure regions, respectively.

We asked whether HBV infection modified the effect of aflatoxin exposure on the proportion of tumors with any mutation in the *p53* gene (*i.e.,* aflatoxin by HBV interaction). We examined this interaction by looking at whether the difference in the proportion of tumors with any *p53* mutation between HBV-positive and HBV-negative tumors changed with aflatoxin exposure. The mean difference in the proportion of tumors that carried any *p53* mutation appeared somewhat larger in the moderate exposure areas (18%) than in either the high (−1%) or the low exposure areas (−3%; Table 2; Fig. 2). However, the mean difference in proportions did not change significantly across aflatoxin levels ($P = 0.15$). Thus, the presence or absence of HBV infection did not appear to modulate the effect of aflatoxin exposure on the prevalence of liver tumors with *p53* mutations. Moreover, the mean difference in proportions between HBV-positive and HBV-negative cases, averaged across aflatoxin levels, was only 5% (95% CI, −6–15%; $P = 0.39$), indicating little or no effect of HBV itself on the proportion of liver tumors carrying *p53* mutations.

Lastly, we investigated whether HBV infection modified the effect of aflatoxin exposure on the proportion of tumors carrying a 249^{ser} mutation. The mean difference in the proportion of tumors with a 249^{ser} mutation between HBV-positive cases and HBV-negative cases was about the same at all of the aflatoxin levels ($P = 0.70$; Table 2). The mean difference in proportions between HBV-positive and HBV-negative cases, averaged across aflatoxin levels, was 6% (95% CI, −1–13%; $P = 0.11$), possibly suggesting a small effect of HBV infection on the proportion of liver tumors with a *p53* 249^{ser} mutation.

Discussion

Accumulation of p53 protein is detectable by immunohistochemistry and is sometimes used as a surrogate for the presence of *p53* mutations because the mutant protein has a longer half-life than the wild type. About half of our samples had accumulation of p53 protein, similar to previous reports from Guangxi (43%; Ref. 53) and Qidong, China (55% and 61%; Refs. 17, 54). However, our results do not support an earlier report that HCC patients with tumors that show p53 protein accumulation are younger than those without it (53).

In vitro experiments (55) have shown that the 249^{ser} mutation may cause loss of tumor suppressor functions in hepatoma cell lines via loss of DNA-binding ability of the p53 protein. A similar finding was described using the mouse *p53*ser246 homologue mutation (28). Experiments with cells from transgenic mice expressing this mutant, under the control of the albumin promoter, have shown that the mutation promotes the transition of hepatocytes from G_0 to G_1 and/or M to G_1. This result suggests a gain of function mutation (56). Interestingly, these transgenic mice had increased liver tumor development when exposed to AFB_1 (57). Enhanced liver tumor development was reduced in mice that also expressed a wild-type *p53*, suggesting that loss of the wild-type allele may be required for the mutant to exert its effect (57). In our study from Guangxi, most tumors with the 249^{ser} mutation did not have a corresponding wild-type allele, suggesting that there had been loss of heterozygosity, resulting in hemizygosity. This observation may support the hypothesis that 249^{ser} mutations are recessive and do not have the dominant negative function reported for some other *p53* mutations.

In vitro studies (27, 58) have shown that AFB_1 can induce the 249^{ser} mutation. These same studies also showed, however, that the 249^{ser} mutation is not the only mutation induced in the *p53* gene by AFB_1, that this codon is not the preferred site for adduct formation, and that the removal of adducts at this site is

neither faster nor more efficient. These findings are not sufficient to explain why a 249^{ser} mutation is more prevalent in high aflatoxin exposure areas.

Our meta-analysis indicates an association between the *p53* 249^{ser} mutation and increasing levels of aflatoxin exposure. This association had been suggested previously by some of the population-based studies carried out in areas with different levels of aflatoxin exposure. However, given the variations in sample sizes, sample origin, and assessment of aflatoxin exposure from study to study, some uncertainty remained. One other meta-analysis (59) of 20 studies has investigated this question but was limited in that it used only two levels of aflatoxin exposure (low and high), included only two studies from high aflatoxin exposure areas, and used statistical methods that did not take into account the sample size of each study. Nonetheless, they also found that areas with high aflatoxin exposure levels were associated with a higher proportion of 249^{ser} mutations in HCC that had *p53* mutations.

Previous reports (18, 24, 32, 39) suggested that 249^{ser} mutation is more common in HBV-positive tumors than in HBV-negative tumors. In the present study, we did not find strong evidence for such an association. The results of our meta-analysis found no clear evidence for an effect of HBV status on the frequency of any *p53* mutation or aflatoxin-HBV interaction on *p53* mutation frequency. We also did not find evidence for an aflatoxin-HBV interaction on 249^{ser} mutation, but we did see some indication, not statistically significant, of a possible effect of HBV on the frequency of this mutation. One of the studies (24) we included in our meta-analysis tested for a possible HBV-aflatoxin interaction using odds ratios of prevalences. In our meta-analysis, we used differences in prevalences instead of odds ratios, as explained in "Materials and Methods." When we used an empirical log odds approach (49) to reanalyze the studies in our meta-analysis, we found weak evidence for an HBV-aflatoxin interaction on 249^{ser} mutations ($P = 0.10$) and significant evidence of an effect of HBV on the frequency of this mutation ($P = 0.005$). However, these results were strongly influenced by one study. Omitting it, we found no evidence for HBV-aflatoxin interaction ($P = 0.57$) and weak evidence for an HBV effect ($P = 0.07$). These results were similar to what we observed using differences in prevalences.

Our meta-analysis has some limitations that are worth discussing. In the majority of the studies, the aflatoxin exposure level was estimated as an ecological measure without using individual measurements of specific biomarkers. This approach may introduce a classification bias because individuals in each study area may vary in their levels of aflatoxin exposure. Regarding HBV exposure, the measurements in all of the studies were done at the individual level. However, two issues should be considered. On the one hand, different methods were used among studies to determine HBV infection status (see "Materials and Methods" for description). These different assays may detect different stages in the infection process that could influence the effect of HBV on *p53* mutations. More specifically, some studies used methods that only detect the HBsAg, which can be absent in subjects that do express the HBV core antigen or that have the *HBV-X* gene integrated in their genome. In our meta-analysis, we did not control for this source of variability. On the other hand, as we showed in our own study in Guangxi, measurements of HBV infection using detection of HBsAg should be carefully done, avoiding the use or pure neoplastic tissue, because that may lead to false-negative results. A few studies included in our meta-analysis assessed HBV status by detecting HBsAg in tumors, and we could not determine whether they had taken this issue into account. Again, this fact might be a source of classification bias.

The results of almost 50 studies of *p53* gene mutations in HCC demonstrate evidence of a dose-response relationship between ecological levels of AFB_1 and prevalence of the *p53* 249^{ser} mutation in primary HCC. Whether AFB_1 causes these mutations or whether AFB_1 exposure leads to differential promotion of cells that acquired the mutation remains unclear. Although many population-based studies have provided evidence that HBV and aflatoxin can synergistically increase HCC risk, we find little evidence that this interaction also occurs at the molecular level in determining the frequency of *p53* mutations.

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